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Systemic RNAi in the small hive beetle (*Aethina tumida* Murray, Coleoptera: Nitidulidae), a serious pest of the European honey bee (*Apis mellifera*)

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Abstract

BACKGROUND: *Aethina tumida* is a serious pest of the European honey bee (*Apis mellifera*) in North America and Australia. Here we investigate whether *Laccase 2*, phenoloxidase gene essential for cuticle sclerotization and pigmentation in many insects, and vacuolar-ATPase *V-type subunit A*, vital for the generation of proton gradients used to drive a range of transport processes, could be potential targets for RNAi-mediated control of *A. tumida*.

RESULTS: Injection of *V-ATPase subunit A* (5 ng) and *Laccase 2* (12.5 ng) dsRNAs resulted in 100 % larval mortality, qPCR confirmed significant decreases and enhanced suppression of transcript levels over time. Oral delivery of *V-ATPase subunit A* dsRNA in solutions resulted in 50 % mortality, however gene suppression could not be verified. We suggest that the inconsistent RNAi effect was a consequence of dsRNA degradation within the gut due to

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the presence of extracellular nucleases. Target specificity was confirmed by a lack of effect on survival or gene expression in honey bees injected with *A. tumida* dsRNAs.

CONCLUSIONS: This is the first study to show evidence for systemic RNAi in *A. tumida* in response to injected dsRNA but further research is required to develop methods to induce RNAi effects via ingestion.

Key words: systemic RNAi, small hive beetle (*Aethina tumida*), *Laccase 2*, *V-ATPase subunit A*, European honey bee (*Apis mellifera*)

1. INTRODUCTION

The European honey bee (*Apis mellifera*) provides essential pollination services to field, horticultural and vegetable crops,¹ as well as hive products like honey, wax, pollen and propolis. Globally, 35 % of crops at an estimated value of €153 billion depend on animal pollinators.^{2,3} The health and vigour of honey bee colonies are threatened by numerous parasites and pathogens.⁴ *Aethina tumida* is a coleopteran parasite that has become a major problem through extension of its geographical range. In its native range of sub-Saharan Africa *A. tumida* is considered an occasional parasite and scavenger of colonies of African honey bee, *A. mellifera scutellata*⁵⁻⁷ and the Cape honey bee, *A. mellifera capensis*.⁸ Growth in the international trade of honey bees and hive products over the past 20 years has enabled the spread of *A. tumida* into several countries, and establishment in North America and Australia has resulted in severe economic damage to the apiculture industry.^{9,10} *Aethina tumida* have also been detected in Egypt (2002), Canada (2002 and 2006), Mexico (2007), Sudan (2007) and Hawaii (2010).¹¹⁻¹³ *Aethina tumida* is a notifiable pest in the UK and

Europe and to-date has not been detected in the UK.¹⁴ This was also the case in Europe until 2014 when *A. tumida* was detected in south west Italy.¹⁵ Since 2011, there have been substantial numbers of bee and queen imports from Italy into the UK, raising potential for an *A. tumida* outbreak. Furthermore, the climate and soils (in many areas) of the UK meet the developmental needs of *A. tumida* and thus there is potential for rapid establishment.¹⁶

Current measures used against *A. tumida* in the USA are often inadequate suffering variability in levels of pest control. The in-hive organophosphate, CheckMite + StripsTM (10 % w/w Coumaphos) designed to control the adult stage are routinely used in conjunction with GardStar7 (40 % permethrin), a soil treatment product aimed to control this pest as “wandering” larvae (i.e. in search of a suitable site for pupation) leave the hive and enter the soil to complete their life-cycle.^{17,18} Organophosphates are highly toxic to bees, wildlife and humans,¹⁹ and hence all hive honey combs have to be removed prior to treatment. Additionally, the continued use of pyrethroids such as permethrin can give rise to resistance, and upon contact, is deleterious to honey bees.²⁰ Given these issues alternative control strategies are urgently required.

RNA interference (RNAi) has been widely used as a means to elucidate gene function and is increasingly being recognised as having potential application for the control of insect pests, as high sequence specificity predicts negligible effects on non-target organisms.²¹⁻²³ This highly conserved eukaryotic post-transcriptional gene silencing mechanism is thought to have evolved as a defence against viruses and transposable elements, and as a means of regulating endogenous gene expression.^{24,25} Environmental RNAi can be induced by introducing double-stranded RNA (dsRNA) into an insect via microinjection, ingestion or soaking, leading to down-regulation of the transcript levels of the targeted gene. The RNA pathway is initiated by the cleavage of dsRNA into short interfering RNA (siRNA) by the nuclease Dicer.²⁶ The siRNA then binds to the RNA induced silencing protein complex in

conjunction with the Argonaute protein, which leads to the specific suppression and degradation of targeted mRNA.²⁵

Whilst the core RNAi machinery appears to be conserved amongst insects, sensitivity to environmental RNAi is highly variable between insect species, developmental stages, tissues and target genes.²² Three processes are thought to facilitate an RNAi effect: cellular uptake of dsRNA, production of secondary dsRNAs in the cell and the transfer of these molecules to other cells. If the RNAi signal spreads to other cells the RNAi effect is considered to be systemic. The precise mechanisms responsible for the cellular uptake of exogenous dsRNA and/or spreading of the silencing signal, both likely to play a key role in determining levels of gene silencing, remain undefined in insects.

RNAi efficiency is also influenced by the mode of delivery, with microinjection allowing known doses of dsRNA to be delivered directly into the haemocoel, enabling access to haemocytes, fat bodies, epidermal cells and the basal gut epithelial membrane, providing the most consistent results. Oral delivery via incorporation into diet or *in planta* expression has proved more challenging and most reported studies have used dsRNAs targeting genes expressed in gut cells, thus avoiding reliance upon the transfer of the silencing signal to distant tissues for gene suppression. Nevertheless, orally induced RNAi has been achieved in Coleoptera,²⁷ Lepidoptera^{27,28} and Hemiptera.^{29,30} Oral delivery of dsRNA to dipteran species has proved more challenging than microinjection, and certain lepidopteran species require high oral doses of dsRNA to trigger an RNAi effect.³¹ Recent studies suggest that variability in RNAi effects may also be influenced by stability of dsRNA to degradation by extracellular nucleases (reviewed by Gu and Knipple²², Scott *et al.*²³).

Evidence for systemic RNAi in *Tribolium castaneum* and successful RNAi studies in other coleopteran insect pests^{32,27} formed the rationale for our investigations into the potential

use of RNAi as a target specific control strategy for *A. tumida*. The phenoloxidase gene *Laccase 2* which plays an essential role in cuticle sclerotization and pigmentation in many insects, and vacuolar-ATPase *V-type subunit A*, a component of an enzyme complex vital for the generation of proton gradients used to drive a range of transport processes, were selected as target genes on the basis of previous RNAi studies.^{27, 32-35} To our knowledge this is the first study to provide evidence for target specific systemic RNAi in *A. tumida*.

2. EXPERIMENTAL METHODS

2.1 Insects

Aethina tumida cultures were maintained in the Quarantine Entomology Unit (Fera Science Ltd.) and were originally established from wandering larvae imported under three levels of containment supplied by the Plant Protection Research Institute, South Africa. Cultures were maintained at 20°C, 65 % RH, under darkness.

Apis mellifera adults and pupae were supplied from the Fera Home Apiary and were maintained at 34°C, 65 % RH, under darkness during bioassays.

2.2 Molecular cloning of *Laccase 2* and *V-ATPase subunit A*

Total RNA was isolated from 3 week old pupae (*Laccase 2*) and wandering *A. tumida* larvae (*V-ATPase subunit A*), respectively, using SV Total RNA Isolation System (Promega) according to manufacturer's instructions. First-strand cDNA was synthesised from 1 µg total RNA in a 20 µl reaction using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with 500 ng oligo(dT)18 primer, according to manufacturer's guidelines. Degenerate

primers were designed to amplify PCR products of 629 base pair (bp), 618 bp and 547 bp for *Laccase_2*, *V-ATPase subunit A* and *GAPDH*, respectively (Table 1). *Laccase 2* degenerate primers were designed based on conserved regions in *T. castaneum* (GenBank accession no. AY884061.2), *Monochamus alternatus* (accession no. EU093075.1) and *Bombyx mori* BmLac2 (accession AB379590.1). *V-ATPase subunit A* degenerate primers were designed based on conserved regions in *T. castaneum*, (accession no. XM_971095.2), *Musca domestica* (accession no. XM_005179917.1) and *Ceratitis capitata* (accession no. XM_004533325.1). PCR reactions were performed using *Taq* DNA Polymerase (Fermentas, Life Technologies) under standard conditions. Amplified products were cloned into pJET1.2 (CloneJET PCR Cloning kit, Thermo Scientific Life Technologies) as described in the manufacturer's protocol. Purified plasmids were sequenced by Eurofins MWG (Ebersberg, Germany). Genbank accession numbers for cloned partial *Laccase 2*, *V-ATPase subunit A* and *GAPDH* sequences are KU696310, KU696311 and KU696309, respectively.

2.3 Production of dsRNA

Target templates for *in vitro* transcription were generated using gene specific primers based on cloned *A. tumida* sequences (Table 1). PCR was conducted using Phusion® High-Fidelity DNA Polymerase (Fermentas, Life Technologies) under standard conditions. Products of 301 bp (*Laccase 2*) and 305 bp (*V-ATPase sub-unit A*) were restricted with *XhoI* and *XbaI*, ligated into plasmid Litmus28i (New England BioLabs) and purified plasmids were verified by DNA sequencing. *Laccase 2* and *V-ATPase subunit A* dsRNAs were prepared using Megascript T7 transcription kit (Ambion), according to the manufacturer's instructions. For control treatments dsRNA was prepared corresponding to a region of a bacterial *nptII* resistance gene (*nptII*). T7-RNA polymerase was used in transcription reactions, with target

template linearized with *XhoI* and *XbaI* to generate single-stranded RNA (ssRNA). Each ssRNA was precipitated by adding equal amounts of lithium chloride and nuclease-free water and re-suspended in Ringers solution (125 mM NaCl, 1.5 mM CaCl₂, 5 mM KCl pH 7.31). Finally, equal amounts of ssRNA were added together and annealed by heating the reaction to 80°C and allowing it to cool to room temperature overnight.

2.4 Analysis of gene expression by quantitative PCR

Quantitative PCR (qPCR) was performed on *A. tumida* and *A. mellifera* cDNA and relative expression of *Laccase 2* and *V-ATPase subunit A* was determined using ViiA™ 7 Real-Time PCR System (Life Technologies) with $\Delta\Delta CT$ methodology. In all cases, except for endogenous gene expression experiments, 3 biological replicates containing 5 pooled insects for each target gene and time point were analysed. qPCR primers were designed using Primer express software for real-time PCR v 2 (Applied Biosystems) (Table 1). Reaction mixtures (20 μ l) contained 1x SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma Aldrich), ROX as a reference dye, 10 μ M qPCR primers and 200 ng of cDNA or water as a negative control. Reactions were run in triplicate. Analysis of amplification profiles was performed using ViiA™ 7 software (Life Technologies), according to the manufacturer's guidelines. qPCR experiments were performed according to the MIQE guidelines outlined by Bustin *et al.*³⁶ Expression of *A. tumida* *Laccase 2* and *V-ATPase subunit A* was normalized to *GAPDH*, whereas *A. mellifera* expression was normalized to Elongation factor-1 (EF-1).³⁷

2.5 Expression of *Laccase 2* and *V-ATPase subunit A* during the life-cycle of *Aethina tumida*

Total RNA was isolated at different developmental stages (eggs, larvae, wandering larvae, prepupae, pupae, non-emerged adult and emerged adult) and first-strand cDNA was

synthesised as stated in section 2.2. In this case a single biological replicate containing 5 pooled insects or 50 mg wet weight of eggs were analysed in triplicate. Relative expression of targeted genes during the life-cycle of *A. tumida* was analysed using qPCR.

2.6 RNAi experiments

2.6.1. *Aethina tumida* injection bioassays

Wandering or 7 day old *A. tumida* larvae were injected using a Hamilton micro-syringe fitted with a 26 gauge needle (Essex Scientific Laboratory Supplies Ltd) with doses ranging from 2-500 ng of *Laccase 2* or *V-ATPase subunit A* dsRNAs; *nptII* dsRNA or Ringers solution served as negative controls. *A. tumida* larvae were anaesthetised using CO₂ and injected with 1 µl (wandering larvae) or 0.5 µl (7 day old larvae) of dsRNAs or Ringers solution. Larvae were injected in the 3rd dorsal segment and needles were left in the larvae for 30 s prior to withdrawal, to reduce the expulsion of fluid from the wound. Larvae were placed in a petri dish after injection to allow the wound to seal. Thereafter, wandering larvae were placed in tubs of sand and monitored for phenotype and/or emergence over a period of 35 days (n=10 per treatment) or removed after 48 hr, 1 week (*V-ATPase subunit A*) or 3 weeks (*Laccase 2*) (n=15 per treatment) for qPCR analysis. Seven day old larvae were treated in the same manner, although after injection they were returned to sandwich boxes containing brood food and left to feed until they entered the wandering stage. Time points for qPCR analysis were selected based on preliminary assays which indicated that insects injected with dsRNAs were still alive at the time of sampling.

2.6.2 *Aethina tumida* feeding bioassays

Egg slides from *A. tumida* were placed onto artificial diet containing 50 % (v/w) aqueous honey solution (1 ml) and 2.5 g of crushed bee pollen. Larvae were allowed to feed for 7 days before being transferred to artificial diets containing *Laccase 2*, *V-ATPase subunit A*, control *nptII* dsRNA or Ringers solution. Thirty larvae per treatment were placed onto 900 mg of diet containing 30 µg of dsRNA (33 ng/mg), and fresh diet was provided after three days. After 6 days of feeding the wandering larvae were placed in tubs of sand and monitored for phenotype and/or emergence over a period of 35 days. For qPCR analysis 15 larvae (per treatment and time point) were treated as stated above. Samples were taken 48 hr after feeding on dsRNA or removed after 1 week (*V-ATPase subunit A*) or 3 weeks (*Laccase 2*) after the wandering stage had commenced.

In a second feeding bioassay 7 day old larvae (n=20) were transferred into sterile falcon tubes containing 300 µl of 50 % (w/v) sterile sucrose solution (prepared with Ringers solution) containing 30 µg of each dsRNA or sucrose solution serving as a negative control. After 24 hr the larvae were transferred to artificial diet to feed until wandering, thereafter the larvae were placed in tubs of sand and monitored for phenotype and/or emergence. qPCR analysis was conducted only for *V-ATPase subunit A* dsRNA treated larvae (n=15 per treatment) with samples being removed after 1 week.

2.6.3 *Apis mellifera* injections bioassays

Newly emerged *A. mellifera* workers were anesthetized by cooling on ice and subsequently injected under the 5th abdominal segment with 2 µl containing 50 ng of *A. tumida* *Laccase 2*, *V-ATPase subunit A*, control *nptII* dsRNAs or Ringers solution. Injections were conducted using a Hamilton micro-syringe fitted with a 33 gauge custom fine needle

(Essex Scientific Laboratory Supplies Ltd). Following injection, worker bees were grouped in cohorts of 10 or 15 individuals, supplied with 50 % (w/v) sucrose solution and placed in an environmental chamber (night cycle, 34°C, and with 60 % R.H). Thereafter, worker bees were monitored for phenotype (n=20 per treatment) for 10 days or removed after 48 hr and 1 week (n=15 per treatment) post-injection for qPCR analysis. Additionally qPCR analysis was carried out on 2 day old pharate adults (part of the pupal stage) injected with *Laccase 2* dsRNA as, according to Elias-Neto *et al.*³⁸ this gene is significantly up-regulated at this stage in the life-cycle. Pharate adults were injected as previously described and after injection individuals (n=15 per treatment) were carefully positioned in a well of a microtiter plate (Thermo Scientific) and removed from the environmental chamber after 48 hr for qPCR analysis. All samples for qPCR analysis were snap frozen in liquid nitrogen and stored at -80°C until use.

2.7 dsRNA stability assays

2.7.1 Persistence of dsRNA in sucrose solutions containing *Aethina tumida* larvae

The stability of dsRNA in sucrose solution was evaluated by incubating 1 µg of *V-ATPase subunit A* dsRNA in 10 µl of 50 % (w/v) sucrose solution at 20°C for 22 hr. Following confirmation that dsRNA was stable under these conditions two 7 day old larvae were incubated in 100 µl of 50 % sucrose solution containing 10 µg of *V-ATPase subunit A* dsRNA, with 10 µl aliquots taken at the following time points: 0, 1, 2, 4, 6, 8, 18 and 22 hr. The integrity of the dsRNA was analysed by separation on 1.2 % (w/v) agarose gels and bands were visualised by ethidium bromide staining under UV.

To determine whether *A. tumida* larvae produced extracellular ribonucleases, 7 day old (i.e. feeding stage) larvae were incubated for 12 hr in sucrose solution as described above.

Thereafter the larvae were removed, 10 µg of *V-ATPase subunit A* dsRNA was added and incubated for 8 hr at 20°C. A second assay was carried out to identify the possible source of ribonuclease activity. Wandering larvae (i.e. non-feeding, cleared guts) and frass were separately incubated for 8 hr, as described previously, in the presence of 10 µg of *V-ATPase subunit A* dsRNA. The integrity of the dsRNA was analysed by agarose gel electrophoresis.

2.7.2 In vitro stability of dsRNA in larval gut extracts

Gut samples dissected from 10 feeding stage larvae were re-suspended in 100 µl Ringers solution and homogenised using a sterile pestle. Protein content was estimated using Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) using Bovine serum albumin as standards. The samples were centrifuged for 5 min at 13 000 rpm and the resulting supernatant was used in the assay. Gut extract samples (10 µg total protein in 20 µl; equivalent to approx. 1/10 of a larval gut) were incubated with 500 ng of *V-ATPase subunit A* dsRNA at room temperature for 5, 15, 30 and 60 min. The integrity of the dsRNA was analysed by agarose gel electrophoresis.

2.8 Statistical analysis

Mortality data from the bioassays were analysed using GenStat version 16.1. Where possible 95 % confidence intervals (C.I) and LD₅₀ values were determined using probit generalized linear regression adjusted for natural mortality. The qPCR results are presented as the mean ± SD of three independent biological replicates and the relative levels of mRNA expression was analysed by One-way ANOVA followed by Tukey test for significant differences between mean values, using GraphPad Prism version 6.00 for windows. $P < 0.05$ was taken as the level of statistical significance.

2.9 CLUSTALW analysis of dsRNA sequences

Aethina tumida dsRNA sequences were compared with *A. mellifera* *Laccase 2* (Genbank: FJ470292) and *V-ATPase subunit A* (GenBank: XM 006567414) using CLUSTALW.

3 RESULTS

3.1 Expression of *Laccase 2* and *V-ATPase subunit A* during development of *Aethina tumida*

As shown in Fig. 1A *Laccase 2* transcripts were detected at significantly higher levels during the three week pupal phase compared to levels at all other developmental stages, although the mRNA was detectable throughout the insect life-cycle. The peak in *Laccase 2* mRNA levels during the third week of the pupal stage coincides with the onset of cuticle tanning, and subsequently declines to a level close to the detection limit in the emerged adult. By contrast, *V-ATPase subunit A* transcripts were readily detectable during all developmental stages (Fig. 1B). Transcript levels were generally higher in the later stages of development (i.e. late larval through pupal stage to adult) with the highest levels detected in wandering larvae and 3 week old pupae.

3.2 Injection of dsRNA to assess phenotype in wandering *Aethina tumida* larvae

The phenotypes observed in wandering *A. tumida* following injections with 500 ng target dsRNAs are shown in Figs. 2A & 2C. All insects injected with *Laccase 2* dsRNA died and were albino-like in appearance, exhibiting a distinct lack of melanisation in comparison to control treatments where tanning was evident 3 weeks after injection (Fig. 2B). Whilst a

phenotype (i.e. lack of melanisation) was evident 3 weeks after injection of *Laccase 2* dsRNA mortality did not occur until 4-5 weeks post-injection and was recorded as failure to emerge from sand as adults at approx. 35 days post-injection. The injection of *V-ATPase subunit A* dsRNA also resulted in a lethal phenotype with treated larvae failing to develop into normal pupae (Fig. 2D). A failure to develop from the wandering to pupal stage was observed approx. 2 weeks after injection of *V-ATPase subunit A* at which point mortality was not always evident; as for *Laccase 2* treated insects mortality was recorded when the controls emerged as adults.

Reducing the dose of *Laccase 2* dsRNA from 500 ng to 12.5 ng did not reduce lethality in wandering stage larvae with 100 % of the adults failing to emerge. A further reduction in injection doses to 10 ng, 5 ng and 2 ng *Laccase 2* dsRNA did provide a dose response, with a respective 90 %, 20 % and 10 % of the adults failing to emerge. *Aethina tumida* injected with *V-ATPase subunit A* dsRNA at concentrations of 12.5 ng, 10 ng and 5 ng resulted in 100 % mortality, with 90 % mortality observed in the 2 ng treatment, assessed as a failure to emerge as adults. Control mortality was 10 % in either Ringers solution or *nptII* dsRNA treatments and 100 % survival was recorded in the non-injected control group (Table. 2). LD₅₀ of 7.49 ng (95 % C.I 2.35-9.35 ng) could only be determined for *Laccase 2* due to the high level of mortality recorded in the *V-ATPase subunit A* treatment.

3.3 Effect of injected dsRNA on gene expression in wandering *Aethina tumida* larvae

To confirm that lethality was a result of a reduction in mRNA levels the expression of target genes in injected insects was assessed by qPCR. For *Laccase 2*, expression levels were analysed for wandering larvae 48 hr and 3 weeks after the injection of 10 ng dsRNA (Fig. 3A). Larvae injected with *Laccase 2* dsRNA exhibited a significant 25-45 % decrease in

Laccase 2 mRNA levels 48 hr post-injection relative to the control groups ($P < 0.01$, One-way ANOVA followed by Tukey test). Analysis of larvae 3 weeks after injection also showed a significant reduction in *Laccase 2* transcript levels (by approx. 70 to 87 %) as compared to the control treatments ($P < 0.0001$, One-way ANOVA followed by Tukey test). Furthermore mean mRNA levels in *Laccase 2* treated insects were significantly lower 3 weeks post-injection as compared to 48 hr post-injection ($P < 0.01$, One-way ANOVA followed by Tukey test). Transcript levels in wandering larvae injected with 2 ng of *V-ATPase subunit* dsRNA were analysed in samples extracted 48 hr and 1 week post-injection (Fig. 3B). A significant 31-54 % decrease in relative levels of *V-ATPase subunit A* mRNA was observed 48 hr post-injection relative to the control groups ($P < 0.001$, One-way ANOVA followed by Tukey test) increasing to 67-85 % in samples taken 1 week after injection ($P < 0.0001$, One-way ANOVA followed by Tukey test). Additionally mean mRNA levels in *V-ATPase subunit A* injected insects were significantly lower 1 week post-injection as compared to 48 hr post-injection ($P < 0.01$, One-way ANOVA followed by Tukey test).

3.4 Injection of dsRNA to assess phenotype and effect on gene expression in 7 day old *Aethina tumida* larvae

Larvae were initially injected in the wandering non-feeding phase as this was an appropriate stage for administering dsRNAs, given endogenous expression of the target genes, and it was also a convenient developmental stage for injection. It has previously been reported that RNAi efficiency can be affected by the developmental stage of an insect.³⁹ To verify persistent and systemic RNAi (prior to oral delivery bioassays) within actively feeding insects, 7 day old larvae were injected with 50 ng of *Laccase 2* and *V-ATPase subunit A* dsRNA. As for wandering larvae, mortality was not evident for *Laccase 2* treated insects until

4-5 weeks post-injection and 2 weeks after injection of *V-ATPase subunit A* dsRNA; and in both cases was recorded as a failure to emerge as adults 35 days post-injection. Control survival ranged from 90-100 % whereas 80 % and 100 % mortality was recorded for *Laccase 2* and *V-ATPase subunit A* treated insects, respectively (Table 2). For insects injected with *Laccase 2* dsRNA tanning was delayed and adults that emerged exhibited developmental abnormalities (Fig. 2B). The transcript levels of *Laccase 2* (assessed 3 weeks after injection of 7 day old larvae with 50 ng dsRNA) were a significant 68-78 % lower than controls (Fig. 3C; $P < 0.0001$, One-way ANOVA followed by Tukey test). For 7 day old larvae injected with 50 ng of *V-ATPase subunit A* dsRNA mRNA levels were significantly reduced (by 72-92 % and 55-90 %, respectively) in samples taken 48 h and 1 week post-injection (Fig. 3D; $P < 0.0001$ and 0.01, One-way ANOVA followed by Tukey test). As observed in insects injected in the wandering phase, development was arrested at the larval stage (Fig. 2D). It is clear from these data that the RNAi effect was persistent and systemic regardless of life stage.

3.5 Oral delivery of dsRNA in artificial diet

To determine if mRNA levels could be down-regulated via oral delivery of dsRNA, 7 day old *A. tumida* larvae (n=30) were fed on artificial diet containing target or control dsRNAs (or Ringers solution as a negative control) for 6 days. Adult emergence was monitored after approx. 35 days; Ringers control emergence was 100 %, whereas 93 % emergence was observed in both *nptII* and *Laccase 2* dsRNA treatments and 73 % emergence was recorded in the *V-ATPase subunit A* dsRNA treatment.

The expression of *V-ATPase subunit A* mRNA in treated insects was assessed by qPCR analysis of larvae collected 48 hr and 1 week after feeding on artificial diets containing dsRNA. Whilst larvae fed on *V-ATPase subunit A* dsRNA exhibited a slight relative decrease

in transcript levels (2-22 %) 48 hr after feeding on dsRNA, no reduction in mRNA levels were observed in samples taken after a feeding period of 1 week (results not shown).

3.6 Stability and oral delivery of dsRNA in sucrose solution

Feeding dsRNA in artificial diets did not trigger RNAi effects and analysis of the stability of dsRNA in the diet proved problematic as separation of the pollen and honey from dsRNA by centrifugation was incomplete and hence it was unclear if the dsRNA remained intact or was degraded over time (results not shown).

Prior to conducting soaking bioassays the stability of dsRNA in the presence of *A. tumida* larvae was assessed by taking samples over a period of 0 to 22 hr. As shown in Fig. 4 the dsRNA remained mostly intact for a period of 1 hr, showing a reduction in size indicative of exonuclease activity. After 2 hr there is approximately half the amount of dsRNA, as compared to time 0, present in the sucrose solution and after 8 hr the dsRNA is completely degraded.

Subsequently a second feeding assay whereby 7 day old larvae (n=20) were soaked for 24 hr (with the solution being renewed at 8 hr intervals) in sucrose solutions containing 100 ng/μl of target or control dsRNA, was conducted. Adult emergence was monitored after approx. 35 days; sucrose and *nptII* dsRNA control emergence was 80 % (n=20) and 82 % (n=17) respectively, whereas 100 % (n=16) emergence was observed in *Laccase 2* dsRNA treatments (Table 3). For *V-ATPase subunit A* dsRNA treatment 50 % (n=18) emergence was recorded, with 17 % of the emerged adults exhibiting morphological deformities (Fig. 5A). However, when this experiment was repeated, qPCR analysis of samples extracted 1 week after feeding on *V-ATPase subunit A* dsRNA showed that transcript levels were significantly

increased rather than decreased in comparison to the control groups (Fig. 5B; $P < 0.05$, One-way ANOVA followed by Tukey test).

3.7 *In vitro* stability of dsRNA in the presence *Aethina tumida* larvae and frass

The secretion of extracellular ribonucleases by larvae was investigated by the addition of dsRNA to a solution after the removal of feeding larvae that had been immersed in sterile water for period of 8 hr. Fig. 6 shows that complete degradation of the dsRNA under these conditions occurs and this is also observed when frass was added to dsRNA containing solutions. By contrast, dsRNA remained intact when wandering (non-feeding) larvae were incubated in dsRNA solutions. These results indicate that extracellular nucleases are secreted as part of the digestive process in the guts of feeding larvae.

3.8 *In vitro* stability of dsRNA in gut extracts

The stability of dsRNA was assessed *in vitro* by incubating dsRNA in gut extracts for 0 to 60 min. Analysis of these samples showed that dsRNA degradation commenced within an incubation period of 5 min and degradation of the dsRNA was complete after 60 min (Fig. 7).

3.9 CLUSTALW analysis of *Aethina tumida* and *Apis mellifera* Laccase 2 and V-ATPase subunit A mRNA

Partial sequences of *A. tumida* and *A. mellifera* Laccase 2 (Genbank: FJ470292) and V-ATPase subunit A (GenBank: XM 006567414) were aligned to assess potential for cross-species RNAi effects. Comparisons of *A. tumida* and *A. mellifera* Laccase 2 and V-ATPase

subunit A mRNAs revealed the presence of conserved regions, however coverage was limited to, at most, a 15 bp region (data not shown). The likelihood of introduced *A. tumida* dsRNA eliciting an RNAi response within the honey bee *A. mellifera* is low given the absence of 20-25 nt stretches of homology.

3.10 Effect of injected *Aethina tumida* dsRNA on phenotype and gene expression in *Apis mellifera*

To investigate whether *A. tumida* target dsRNAs caused mortality and/or down-regulation of *A. mellifera* *Laccase 2* and *V-ATPase subunit A* mRNAs, adult honey bees were injected with 50 ng of target dsRNAs. Survival for both controls and dsRNA treated *A. mellifera* was 100 % after 10 days. As shown in Fig. 8, qPCR analysis confirmed that mRNA levels were not down-regulated in either *Laccase 2* injected pharate adults or *V-ATPase subunit A* injected adult honey bees, as compared to controls. Considerable variation in expression levels across different replicates was notable in these experiments and may, in part, be attributable to slight differences in the developmental stage of the bees that were used in the assays.

4. DISCUSSION

The small hive beetle (*A. tumida*), a scavenger and predator of the European honey bee, has already spread from Africa to countries including the US, Australia, Canada and Mexico and has potential to establish in Europe and the UK. Current pest control measures are challenged by the need for target specificity and high efficacy. RNAi, able to cause the destruction of target specific mRNAs, offers possibilities for the development of a new

approach to combat this economically significant pest without jeopardising the health of honey bee populations.

Here we report significant dose-dependent mortality of *A. tumida* following the injections of 2-12.5 ng doses of dsRNAs targeting *Laccase 2* and *V-ATPase subunit A* mRNAs. Analysis of relative mRNA levels by qPCR confirmed target gene knock-down and significantly enhanced levels of gene suppression over time demonstrated that the RNAi effect was persistent and systemic.

Laccases are a group of multi-copper enzymes present in plants, fungi, bacteria and insects.³⁹ In many insects, two types of laccase genes have been identified, namely *Laccase 1* and *Laccase 2*.^{33,34} *Laccase 2*, a phenoloxidase gene, is expressed in the insect epidermis and has been shown, using RNAi, to be essential for normal beetle cuticle tanning.³³ Injections of dsRNA encoding *Laccase 2* into prepupal *T. castaneum* resulted in dose and time-dependant mortality. Delivery of 200 ng of dsRNA per prepupa inhibited tanning in adults, and resulted in severe developmental abnormalities and mortality. When the dose was reduced to 2 ng per prepupa, this resulted in more normal looking adults, although a degree of malformation was observed and the tanning process was delayed by several days. This is comparable to the results presented in this study where *A. tumida* injected with 500 ng of *Laccase 2* dsRNA showed an albino type appearance, a distinct lack of melanisation and failure to emerge. A further reduction in dose to 12.5 ng and 10 ng, resulted in a similar phenotype and the adult that emerged was distinctly malformed, struggled to walk and died in a premature manner. The observed phenotype was confirmed to be a consequence of down-regulation of *Laccase 2* and, in addition qPCR analysis provided evidence for an increase in levels of gene suppression with time, indicative of transmission and persistence of the silencing signal.

V-type ATPases are highly conserved membrane bound proton pumps responsible for multiple processes including the acidification of organelles (eg. secretory vesicles,

lysosomes) and the maintenance of membrane potential. This enzyme is present in almost all epithelial tissues of insects and plays a vital role in nutrient uptake and ion balance in the insect digestive tract^{40,41} making it an ideal target for RNAi. V-type ATPases are heteromultimeric proteins comprised of a membrane bound protein conducting complex and a peripheral catalytic domain; sub-unit A is one of 8 peripheral domain sub-units that are located on the apical membrane surface. *V-ATPase subunit A* was used as a target gene in the breakthrough RNAi study where it was shown that orally delivered dsRNAs were highly efficacious towards larvae of Western (*Diabrotica virgifera*) and Southern (*D. undecimpunctata howardi*) corn rootworm and Colorado potato beetle (*Leptinotarsa decemlineata*) larvae.²⁷ Here we show that *A. tumida* larvae have a strong RNAi response to *V-ATPase subunit A*, as delivery of as little as 2 ng of *V-ATPase subunit A* dsRNA elicited a lethal phenotype. This phenotype was observed several days prior to pupal metamorphosis when *V-ATPase subunit A* transcript levels are at their highest during the life-cycle of *A. tumida*. Gene expression analysis revealed that larvae injected with *V-ATPase subunit A* dsRNA exhibited significantly enhanced levels of gene suppression from 48 hr to 1 week post-injection. These data, as for *Laccase 2*, indicated that the silencing signal was amplified in *A. tumida* after injection with dsRNA.

Attempts to induce RNAi effects via oral delivery produced highly variable results. A factor that may influence RNAi efficiency is the development stage at which the insect is fed dsRNA.³⁹ Araujo *et al.*⁴³ reported that transcript levels of nitrophorin 2 from the saliva glands in *Rhodnius prolixus* were down-regulated in the 2nd instar relative to controls, however no effect was observed in 4th instar larvae after feeding on artificial diets containing dsRNA. Our results show that injections of *V-ATPase subunit A* dsRNAs resulted in mortality and target gene suppression in both feeding and wandering stage larvae suggesting that life stage is not a limiting factor in eliciting an RNAi response within *A. tumida*.

It is clear that *A. tumida* are highly susceptible to dsRNA delivered via injection, but larvae fed on diets or “soaked” in solutions containing dsRNA showed no consistent evidence for RNAi effects. The complete lack of phenotype or gene suppression observed for feeding experiments with *Laccase 2* dsRNA were not unsurprising given the requirement for an RNAi effect upon delivery of the silencing signal from the gut to the epidermal tissue, where this gene is expressed. By contrast, 50 % mortality and deformities in surviving adults that had been soaked as feeding stage larvae in *V-ATPase subunit A* dsRNA containing solutions was indicative of an RNAi effect. Nonetheless these results could not be validated by qPCR analysis in a repeat experiment. This is in contrast to Baum *et al.*²⁷ who reported that coating synthetic diets with target specific *V-ATPase subunit A* dsRNAs provided respective LD₅₀'s of 1.82 and 5.2 ng/cm² for *D. virgifera* and *L. decemlineata* larvae. However, even in this study no effects on survival or growth were observed when larvae of the cotton boll weevil (*Anthonomus grandis*) were fed on diets coated with dsRNA, leading the authors to suggest that not all coleopteran larvae may be sensitive to orally delivered dsRNA. As for *A. tumida*, the desert locust (*Schistocerca gregaria*) and the migratory locust (*Locusta migratoria*) have been shown to be highly sensitive to dsRNA when delivered via injection, but oral delivery of dsRNA has proved unsuccessful.^{44,45}

We speculated that the lack of consistent effects for *A. tumida* larvae fed on dsRNAs may be a consequence of dsRNA degradation within the gut of *A. tumida* preventing sufficient uptake of dsRNAs by epithelial cells to induce an RNAi response. Initial experiments showing increased dsRNA degradation with time when feeding larvae were soaked in sucrose solutions was indicative of ribonuclease activity, either in the gut or larval regurgitant. *In vitro* studies also provided evidence that dsRNA was prone to degradation with complete digestion occurring within an hour of incubation with gut tissue, although it is noted that these homogenised extracts would contain intracellular and extracellular

ribonucleases. Degradation of dsRNAs by extracellular ribonucleases was illustrated by the instability of dsRNA that had been added to solutions in which larvae had been soaked, and by degradation following the addition of frass to dsRNA containing solutions. By contrast, no degradation was observed when wandering (i.e. not feeding) stage larvae were incubated in dsRNA solutions providing further evidence that extracellular ribonucleases are secreted during digestion in the larval gut. Similarly Allen and Walker⁴⁶ who found that RNAi could be induced by injection but not feeding in the hemipteran plant bug (*Lygus lineolaris*) hypothesised that dsRNA degradation prevented uptake of dsRNA into cells and demonstrated that saliva rapidly digested dsRNA. There is also direct evidence for the expression of dsRNA-degrading enzymes in the digestive juice of larvae of the lepidopteran *Bombyx mori*.⁴⁷ More recently Wynant *et al.*⁴⁵ identified four candidate double stranded ribonucleases (dsRNase) that are expressed in the gut of the locust *Schistocerca gregaria* and subsequently provided evidence for the involvement of *Sg-dsRNases 2* in the degradation of dsRNA. We provide further evidence here to illustrate that the protection of dsRNA from degradation by RNases plays a key role in determining the successful application of RNAi for insect pest control.

The exploitation of RNAi as a strategy for the control of insect pests requires careful selection of target genes in order to achieve specific and effective silencing. Generally a specific segment of mRNA not shared amongst insects is targeted to elicit the RNAi effect in the selected pest. When dsRNAs are introduced into a cell they are cleaved into short fragments of approximately 20-25 nt in length and bind with high specificity to endogenous mRNA, disrupting the expression of the targeted protein product. Baum *et al.*²⁷ reported that *D. virgifera V-ATPase subunit A* dsRNAs produced an effective oral RNAi effect in *D. virgifera* larvae but also (when fed at higher concentrations) in *L. decemlineata*. The nucleotide sequence identities between *D. virgifera* and *L. decemlineata* were 83 % for V-

ATPase subunit A and 3 identical regions of 20-29 nucleotides can be identified in the published sequence alignment providing an explanation for non-target effects. In this study the alignment of *A. mellifera* and *A. tumida* *Laccase 2* and *V-ATPase subunit A* mRNAs indicated at most conserved regions of 15 bp and sequence identities were 74 % and 68 %, respectively. Honey bees are known to be highly susceptible to RNAi.^{48,49} We hypothesised that the absence of identical regions of more than 20 nucleotides in *A. mellifera* and *A. tumida* *Laccase 2* and *V-ATPase subunit A* sequences would ensure that RNAi effects would be specific to *A. tumida*. In agreement we demonstrated that injections of 50 ng of dsRNAs into honey bees had no effect on survival and did not induce suppression of either of the target genes.

Our work has shown that the small hive beetle has a robust and systemic RNAi response to injected, but not ingested dsRNAs, targeting the genes *Laccase 2* and *V-ATPase subunit A*. An absence of effects on survival and gene expression in honey bees injected with *A. tumida* dsRNAs was consistent with target specificity predicted on the basis of sequence alignments of orthologous genes. Whilst oral delivery of *V-ATPase subunit A* dsRNA resulted in increased *A. tumida* larval mortality and malformed survivors, these results could not be verified by qPCR analysis. Evidence for degradation of ingested dsRNAs by extracellular ribonucleases in the guts of feeding larvae is thought to explain, at least in part, the lack of consistency in feeding experiments. The development and implementation of RNAi based pesticides holds great potential for new target specific and environmentally benign applications. However, to translate this approach into a viable control strategy for target specific control of *A. tumida* in apiculture a further research to develop a suitable method to induce an oral RNAi response is required.

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Table 1. Sequence of forward (F)/reverse (R) primers used for both *Laccase 2* and *V-ATPase subunit A* cDNA subcloning, dsRNA synthesis and qPCR analysis

Primer	Sequence 5'-3'
PCR for cDNA sequencing	
<i>Lac</i> F1	GACGTVGAGAACCAYATSGAAGG
<i>Lac</i> R1	CGTATCKTTCMCCWGARAACG
<i>VTE</i> F2	GKGARATYATYCGTYTGARGGYGAHATG
<i>VTE</i> R1	GMYTGYGAGATKACRGTYTTRCCRCA
PCR for dsRNA synthesis	
<i>Lac</i> (RS) F	TATCTCGACGTGGAACCCAATATTACGA
<i>Lac</i> (RS) R	ATATCTAGAGACCGGTGTTTACAGCCAAT
<i>VTE</i> (RS) F	TATCTCGAGGGTGTAACAGTTGGTGATC
<i>VTE</i> (RS) R	ATATCTAGACCCTTGGCTTTAGGTGGCA
Quantitative PCR (qPCR)	
<i>A. tumida Lac</i> F	CCCATTGGAAGTGTTACCAT
<i>A. tumida Lac</i> R	GAAGCGAAGGAGTTGATGATACG
<i>A. tumida VTE</i> F	TGTGGCCTGTACGTCAACCA
<i>A. tumida VTE</i> R	TCCGGTGAGAAGAGGATGATTC
<i>A. tumida GAPDH</i> F	TTCGAGATCGTGGAAGGTTTG
<i>A. tumida GAPDH</i> R	CAGAGGGACCGTCGACAGTT
<i>A. mellifera Lac</i> F	CGTGAGCCAATTGAAGAATG
<i>A. mellifera Lac</i> R	GCCTGTAGAAGAGGAAACGG
<i>A. mellifera VTE</i> F	GACATCGACTTTCTCACC GA
<i>A. mellifera VTE</i> R	AGTAAGCCTTGGCTCGTCAT
<i>A. mellifera EF-1</i> F	CTGGTACCTCTCAGGCTGATTGT
<i>A. mellifera EF-1</i> R	GCATGCTCACGAGTTTGTCCATTCT

Table 2. Survival of *Aethina tumida* wandering and feeding stage larvae (n=10 per treatment) injected with different doses of *Laccase 2* or *V-ATPase subunit A* dsRNAs, controls were injected with *nptII* dsRNA or Ringers solution.

Treatment	Dose (ng)	Survival (%)	95 % C.I (ng)
Wandering stage			
Non-injected	-	100	-
Ringers Control	-	90	-
<i>nptII</i>	12.5	90	-
<i>Laccase 2</i>	12.5	0	2.35-9.35
	10.0	10	-
	5.0	80	-
	2.0	90	-
<i>V-ATPase subunit A</i>	12.5	0	-
	10.0	0	-
	5.0	0	-
	2.0	10	-
95 % C.I survival (%)			
Feeding stage larvae			
Non-injected	-	100	74 – 100
Ringers Control	-	100	74 – 100
<i>nptII</i>	50	90	62 - 99
<i>Laccase 2</i> *	50	20	4 - 50
<i>V-ATPase subunit A</i>	50	0	0 - 26

* Both of the emerged adults had deformities, but were included in the survival %

Table 3. Survival of *Aethina tumida* soaked in 50 % sucrose solutions containing 10 µg of *Laccase 2*, *V-ATPase subunit A* or *nptII* dsRNA and sucrose solution served as an additional control.

Treatment	Survival (%)	Sample No.	95% C.I survival (%)
Sucrose Control	80	20	59 - 92
<i>nptII</i>	82	17	60 - 95
<i>Laccase 2</i>	100	16	83 - 100
<i>V-ATPase subunit</i>	50	18	28 - 72

* 3 of the emerged adults had deformities, but were included in the survival

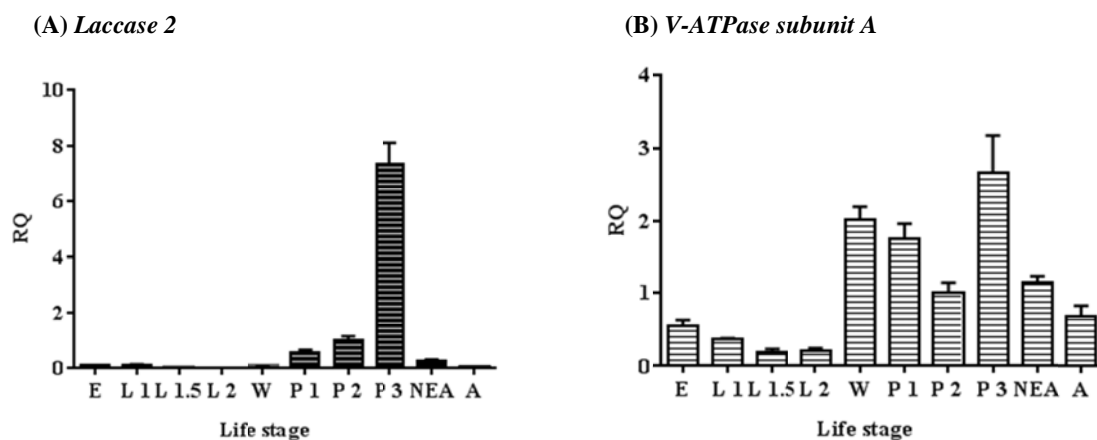


Figure 1. Expression of: (A) *Laccase 2* and (B) *V-ATPase subunit A* genes throughout the life-cycle of *Aethina tumida*, assayed by quantitative PCR. Developmental stages as follows: E denotes eggs; L1-W, different stages of larval growth (1, 1.5 & 2 week old larvae; W=wandering stage); P1-P3 are samples taken at 1, 2, and 3 week intervals during the pupal stage; NEA are non-emerged adults and A are emerged adults. Expression levels are normalised to *GAPDH* mRNA; RQ was set to 1 for P2 stage samples. All error bars represent the \pm SD of the mean, as determined from one replicate (n=5 per insect replicate or 50 mg wet weight of eggs), each with three technical replicates.

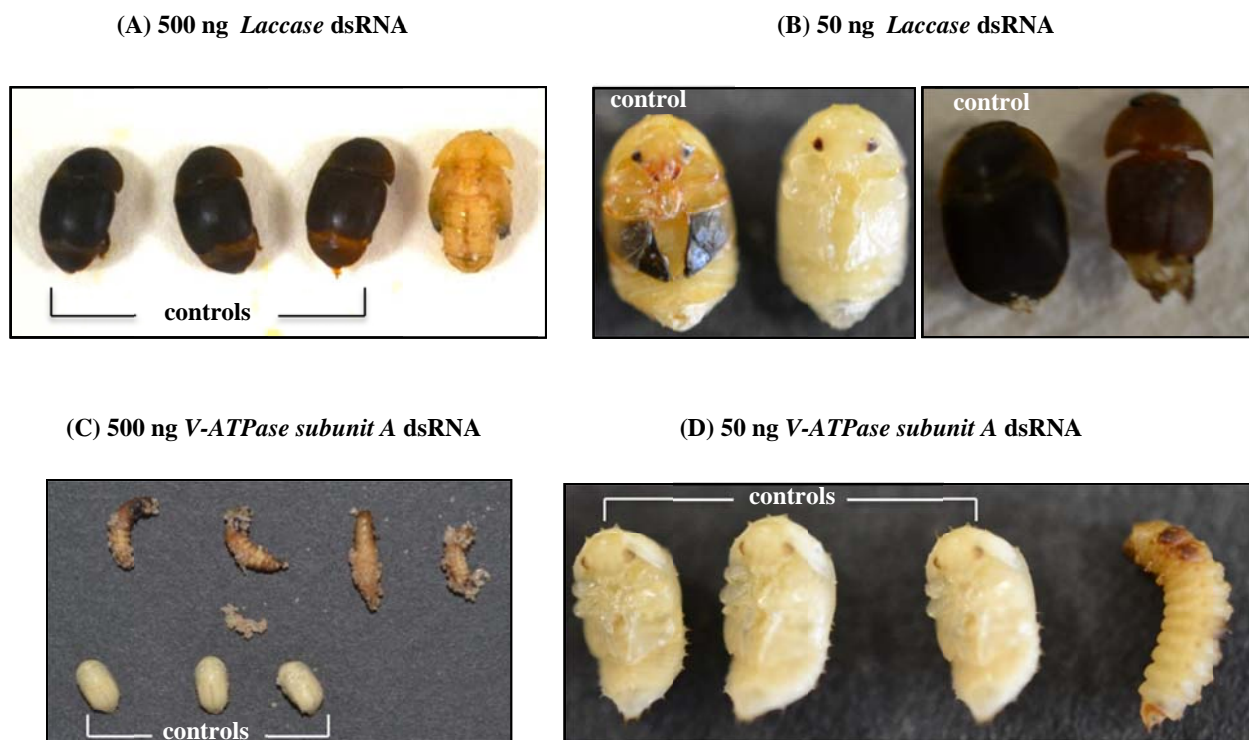


Figure 2. *Aethina tumida* phenotype after injection with dsRNAs: (A) Wandering stage larvae injected with 500 ng *Laccase 2* dsRNA, controls from left to right are not injected; injected with Ringers solution and *nptII* dsRNA (35 days post-injection); (B) Feeding stage larvae injected with 50 ng *Laccase 2* dsRNA (left 17 days and right 35 days post-injection); (C) Wandering stage larvae injected with 500 ng *V-ATPase subunit A* dsRNA (14 days post-injection), controls from left to right are as denoted in (A) (post-injection); (D) Feeding stage larvae injected with 50 ng *V-ATPase subunit A* dsRNA, controls from left to right are as denoted in (A) (14 days post-injection).

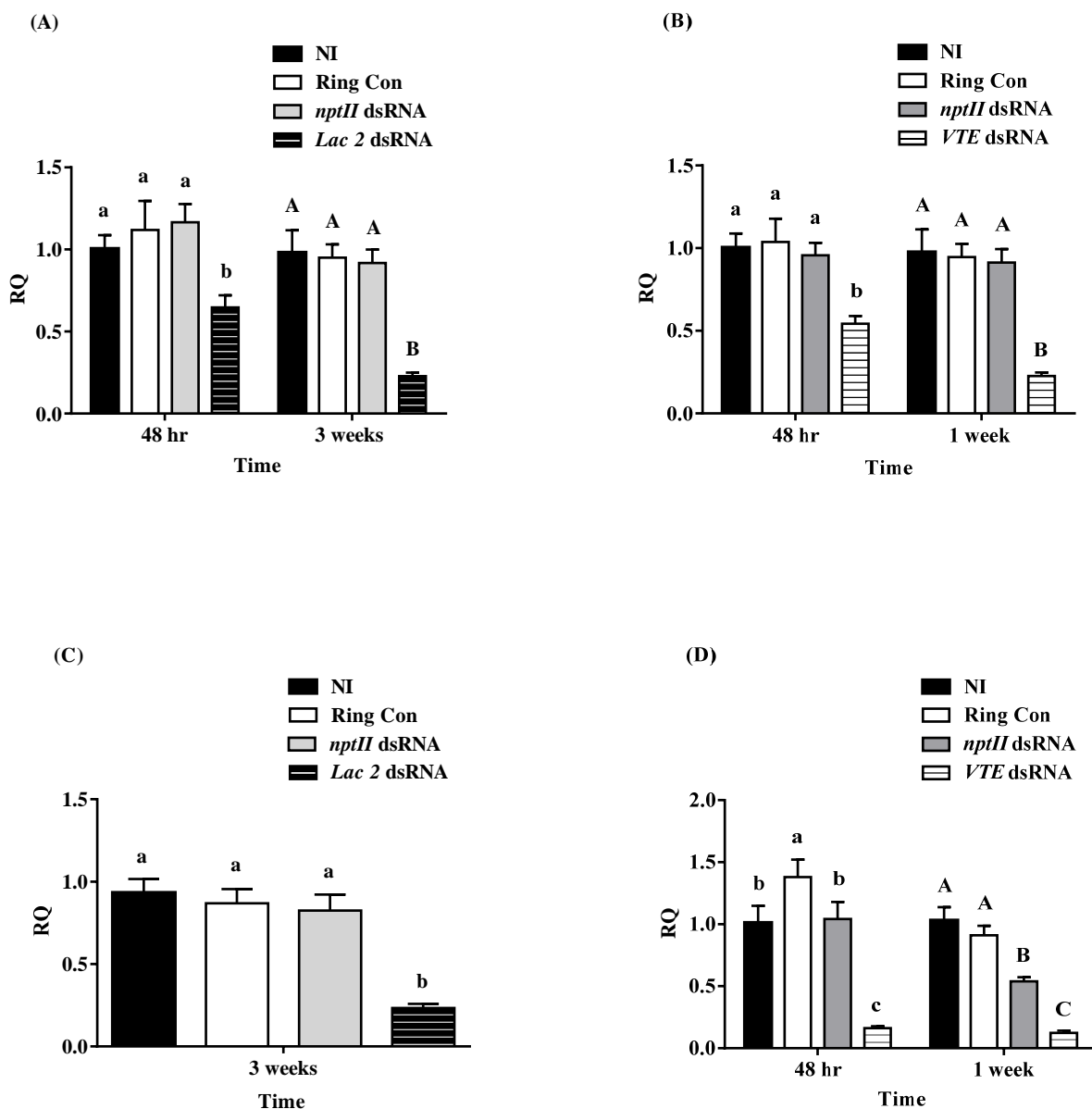


Figure 3. Relative expression of: (A) *Laccase 2* mRNA in *Aethina tumida* wandering larvae 48 hr and 3 weeks after injection of 10 ng *Laccase 2* dsRNA (*Lac 2*); (B) *V-ATPase subunit A* mRNA in wandering larvae 48 hr and 1 week after injection of 2 ng *V-ATPase subunit A* dsRNA (*VTE*); (C) *Laccase 2* mRNA in feeding stage (7 day old) larvae 3 weeks after injection of 50 ng *Laccase 2* dsRNA; (D) *V-ATPase subunit A* mRNA in 7 day old larvae 48 hr and 1 week after injection of 50 ng *V-ATPase subunit A* dsRNA. Controls are non-injected (NI); Ringers (Ring Con) and *nptII* dsRNA injected. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for NI. All error bars represent the \pm SD of the mean, as determined from three independent replicates ($n=5$ insects per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

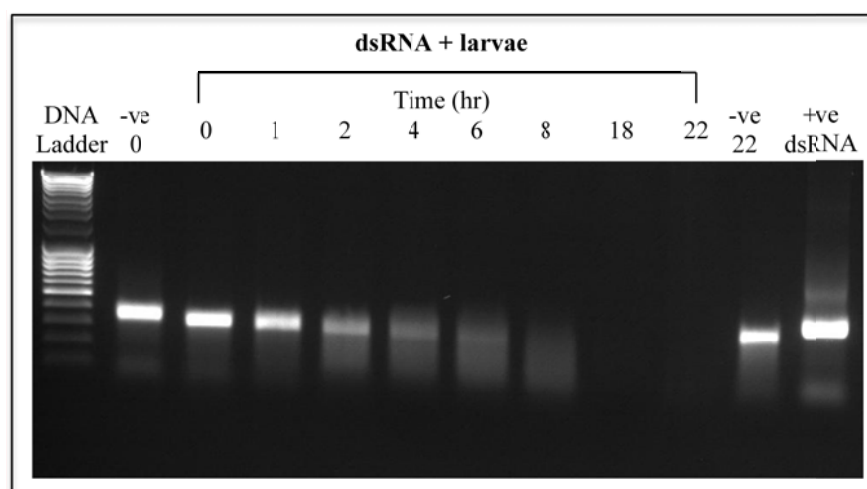


Figure 4. Stability of dsRNA in the presence of feeding stage *Aethina tumida* larvae. Larvae were immersed in sterile sucrose solution containing dsRNA and samples taken at specified time points were analysed for integrity by agarose gel electrophoresis. Negative controls are –ve 0 and –ve 22 which show dsRNA with no larvae present at time 0 and after 22 hr; +ve dsRNA denotes dsRNA re-suspended in Ringers solution.

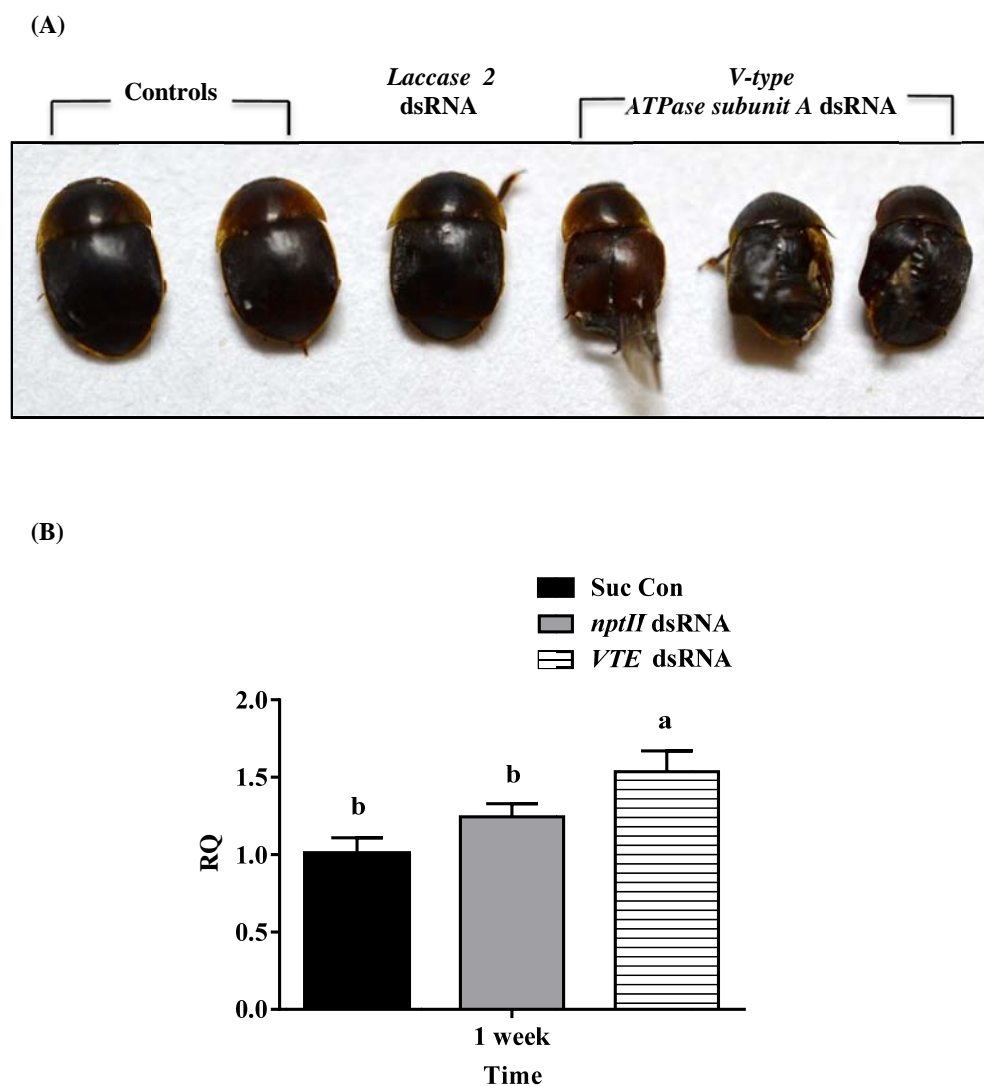


Figure 5. (A) Adult *Aethina tumida* 35 days after soaking for 24 hr in sucrose solutions containing 0.1 $\mu\text{g}/\mu\text{l}$ of *Laccase 2* dsRNA or *V-ATPase subunit A* dsRNA; controls left and right are sucrose only treated and *nptII* dsRNA treated; (B) Expression of *V-ATPase subunit A* mRNA in *A. tumida* after soaking for 24 hr in sucrose solutions containing 0.1 $\mu\text{g}/\mu\text{l}$ of *V-ATPase subunit A* dsRNA (*VTE*), samples were removed 1 week after feeding. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for sucrose soaked control sample

(Suc Con). All error bars represent the \pm SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

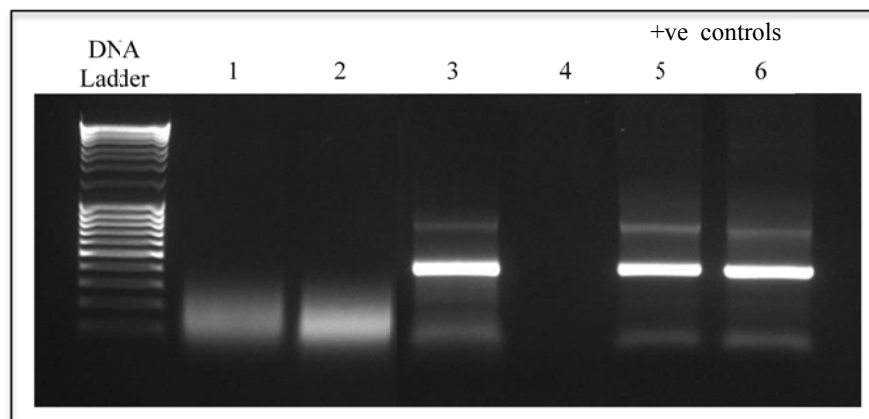


Figure 6. Stability of dsRNA in the presence of *Aethina tumida* larvae and frass. All samples were incubated for a period of 8 hr and analysed by agarose gel electrophoresis. Lane 1, 10 kb DNA ladder; lane 2, dsRNA added after the removal of 7 day old (feeding) larvae; lane 3, feeding larvae incubated with dsRNA; lane 4, wandering larvae incubated with dsRNA; lane 5, larval frass incubated with dsRNA, lane 6 and lane 7, positive (+ve) controls denoting dsRNA added to 50 % sucrose solution and dsRNA alone, respectively.

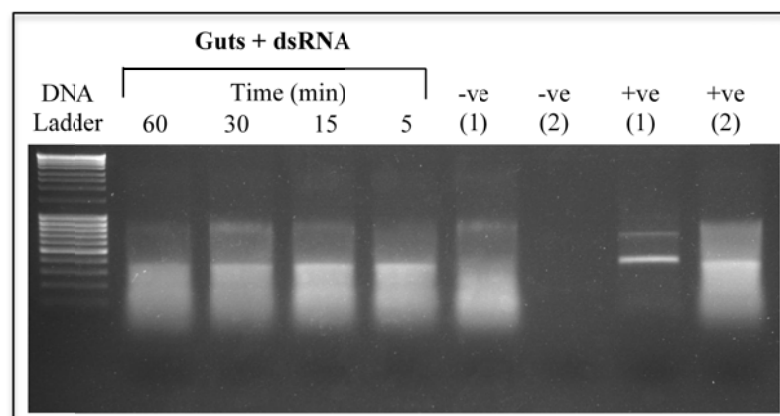


Figure 7. *In vitro* stability of dsRNA in *Aethina tumida* larval gut extracts. Samples were taken at indicated time points after incubation of 500 ng dsRNA with gut extract (approx. 1/10 of a larval gut; final volume 20 μ l) and analysed by agarose gel electrophoresis, 20 μ l was loaded in all lanes. Negative controls are (1) gut sample alone, and (2) Ringers solution. Positive controls are (1) dsRNA re-suspended in Ringers solution and (2) dsRNA and gut extract at 0 hr.

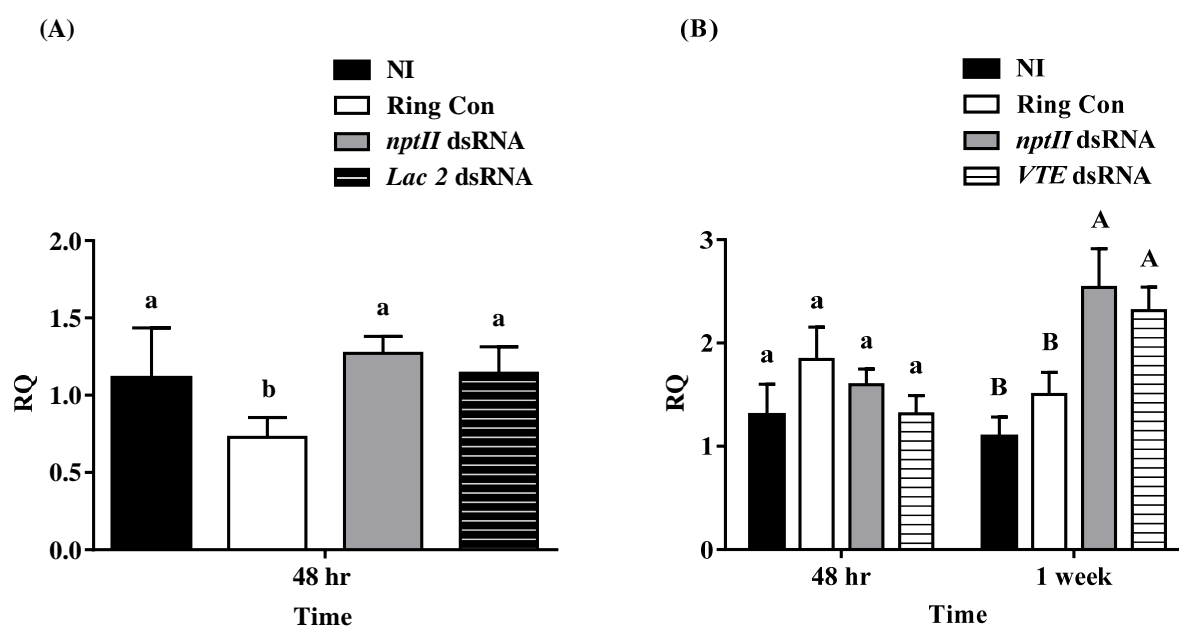


Figure 8. Relative expression of: (A) *Laccase 2* mRNA in 2 day old *A. mellifera* pharate adults 48 hr after injection with 50 ng of *A. tumida* *Laccase 2* dsRNA (*Lac 2*); (B) *V-ATPase subunit A* mRNA in *A. mellifera* 48 hr and 1 week post-injection with 50 ng of *A. tumida* *V-ATPase subunit A* dsRNA (*VTE*). Expression levels are normalised to Elongation factor-1 (EF-1) mRNA. Controls are non-injected (NI); Ringers (Ring Con) and *nptII* dsRNA injected. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for NI. All error bars

represent the \pm SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.